Epidermolysis Bullosa Acquisita Antigen, a New Major Component of Cutaneous Basement Membrane, Is a Glycoprotein with Collagenous Domains

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The epidermolysis bullosa acquisita antigen is a major constituent of the basement membrane zone beneath stratified squamous epithelium. The antigen which is recognized in extracts of skin basement membrane by Western blot analysis with polyclonal or monoclonal antiepidermolysis bullosa acquisita antigen antibodies as 2 chains (a major chain of 290,000 and a minor chain of 145,000) has a native molecular weight over 800,000. Both epidermolysis bullosa acquisita antigen chains contain carbohydrate and the 290K chain is sensitive to collagenase. *J Invest Dermatol 86: 668–672, 1986*

Epidermolysis bullosa acquisita (EBA) is a severe subepidermal blistering disease of the skin [1]. Patients with EBA have immune complexes at the basement membrane zone (BMZ) beneath the epidermis, the so-called dermal-epidermal junction [2,3]. By direct immunoelectron microscopy, these immune products are localized precisely to an area within the BMZ immediately below the lamina densa [2,3]. Moreover, many patients with EBA have anti-BMZ autoantibodies in their sera that react with the cutaneous BMZ when examined by indirect immunofluorescent or indirect immunoelectron microscopy [2–4]. The target for these circulating antibodies is within and immediately below the lamina densa, the site of the blister cleavage plane [2–4]. We have shown previously that the antigenic target in the cutaneous BMZ of human skin for the autoantibodies in the sera of EBA patients is an extracellular matrix molecule identified as 2 protein chains: a major chain of 290,000 daltons and a minor chain of 145,000 daltons [4]. Further, this new BMZ component is synthesized by human keratinocytes [5] and fibroblasts [6,7].

In this report, we present evidence that the "EBA antigen," a major component of the BMZ beneath stratified squamous epithelia, is a glycoprotein with a native molecular weight over 800,000 and has collagen-like domains.

MATERIALS AND METHODS

Preparation of Human Skin BMZ Extract Adult human skin was obtained from surgical specimens or amputated limbs, and the BMZ extracted with 8 M urea, 0.3 M mercaptoethanol in 0.05 M Tris-HCl buffer, pH 8.6, as previously described [4] for 1.5 h at room temperature.

Polyacrylamide Gel Electrophoresis and Western Blots The extracted proteins were separated by polyacrylamide gel electrophoresis on a 5% polyacrylamide-sodium dodecyl sulfate (SDS) gel as described by Laemmli [8], transferred to nitrocellulose paper as described by Towbin et al [9], and reacted with EBA patient sera diluted 1:200 in 0.01 M Tris-HCl, 0.25 M NaCl, pH 7.2, 0.3% Nonidet P-40, 3% bovine serum albumin (TNB buffer) or with a murine monoclonal antibody, H3a, to the EBA antigen as previously reported [4,10].

Identification of EBA Antigen Chains in Gels Stained for Protein and Carbohydrate The BMZ extracted proteins (50 μg/lane) were separated by polyacrylamide gel electrophoresis and stained for protein with 0.1% Coomassie blue as described by Laemmli [8] or for carbohydrate with a 1% metabisulfite-fuchsin stain as described by Zacharius et al [11]. Protein and carbohydrate bands with relative migrations identical to the bands reacting with EBA antibodies in the Western blot experiments were identified. These stained bands were cut out of the gel and reelectrophoresed into a second 5% polyacrylamide gel according to Cleveland et al [12]. The reelectrophoresed bands were then either stained for protein (Coomassie blue), or carbohydrate (metabisulfite-fuchsin) or subjected to Western blot analysis with EBA antibodies. Laminin (30 μg/lane) isolated from a transplantable murine tumor as previously described [13,14] was used as a positive control for all gels stained with metabisulfite-fuchsin.

Proteolytic Domains of EBA Antigen Chains The 290 kD and 145 kD EBA antigen bands were cut out of a 5% polyacrylamide-SDS gel, equilibrated in 0.25 M Tris-HCl, 0.1% SDS, pH 6.8, and then reelectrophoresed into a second slab gel in the presence of varying amounts (0, 0.0025 μg, 0.025 μg, 0.25 μg, or 0.5 μg) of chymotrypsin (Miles, Elkhart, Indiana) or staphylococcal V-8 protease enzyme (Miles) as described by Cleveland et al [12]. At the completion of the second electrophoresis, the gels were stained for protein with Coomassie blue [8] or silver nitrate according to Wray et al [15] or the proteins were transferred to nitrocellulose paper and reacted with EBA antibodies by Western blot analysis [4,9].

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Abbreviations:

BMZ: basement membrane zone
EBA: epidermolysis bullosa acquisita
SDS: sodium dodecyl sulfate

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Collagenase Sensitivity of EBA Chains Three milligrams of lyophilized BMZ extract or bovine serum albumin (Miles) were placed into 1500 μl of 0.025 M Tris-HCl, 0.01 M calcium acetate, 1% bovine serum albumin, 0.001 M phenylmethyl-sulfonyl fluoride, 0.04 M N ethylmaleimide, pH 7.2 (CAB) and divided into 250-μl aliquots. Proteinase-free collagenase (2.5 or 5.0 units; Advanced Biofactures-Type III, Lynbrook, New York) or 5.0 units of boiled collagenase were added to each tube, gently agitated, and incubated at 37°C for 1 h. The tubes were placed on ice and 500 μl of CAB added in addition to 80 μl of 0.1 M EDTA. The samples were dialysed against distilled water at 4°C and lyophilized. The lyophilized samples were brought up to 0.025 M sodium phosphate, 2% SDS, pH 6.8, to a concentration of 1 mg/ml and 40 μg/lane electrophoresed on a 7.5% polyacrylamide-SDS slab gel as described above. The proteins were then subjected to Western blot analysis against anti-EBA antigen antibodies [4] or antibovine serum albumin antibodies (Cappel, Cochranville, Pennsylvania) diluted 1:400 in TNB buffer.

Elution of EBA Antigen from Gels and Iodination The 290 kD and 145 kD bands of the BMZ extract were cut out of Coomassie blue-stained 5% polyacrylamide-SDS slab gels. Five bands of each chain were eluted from the gel slices as described by Hunkapillar et al [16]. By Lowry protein assay [17] approximately 40 μg of the 290 kD band and 186 μg of the 145 kD bands were recovered. Twenty micrograms of each band were subjected to labeling with [^{125}I]sodium iodide (Amersham, Arlington Heights, Illinois) by the chloramine-T reaction [18].

RESULTS

The skin BMZ extract consisted of multiple protein bands. However, by Western blot analysis only 2 of the bands (M_r = 290,000 Kd and M_r = 145,000) labeled with antibodies from the sera of EBA patients (Fig 1) or the H_3a monoclonal antibody (not shown).

Both of the EBA chains stained for carbohydrate when the gels were stained with metabisulfite-fuchsin. However, there was a much greated degree of staining of the 145 kD chain than the 290 kD chain. It is interesting that the 2 EBA chains were the 2 bands in the extract that had the most prominent staining for carbohydrate (Fig 2). To show definitively that the EBA chains were the bands containing carbohydrate in the BMZ extract, the 290 kD and 145 kD bands (migrating identically to bands reacting with EBA antibodies in the Western blots) were cut out of a 5% polyacrylamide gel and then reelectrophoresed into a second gel and stained for carbohydrate or reacted with EBA antibodies in Western blots. To enhance the visualization of carbohydrate in the 290 kD chain, 2 identical bands of the 290 kD chain, and 1 band of the 145 kD chain were reelectrophoresed. The second gel of the reelectrophoresed EBA chains shows approximately equal staining intensity of the 2 lanes (Fig 3), confirming that there is carbohydrate in the 290 kD chain but less than that in the 145 kD chain. The reelectrophoresed and transblotted bands reacted with monoclonal and polyclonal EBA antibodies in the Western blots and showed identical migrations to the bands in the whole extract that react with these antibodies. Therefore, in addition to showing that these bands contain carbohydrate, these experiments also confirm that the bands in the BMZ extract migrating at 290

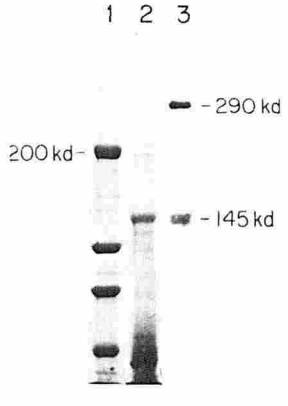

Figure 1. A Western blot of skin basement membrane extract reacted with antibodies from the serum of a patient with EBA. *Lane 1* is a high-molecular-weight standard transferred to nitrocellulose and stained for protein with amido black. *Lane 2* is an amido black-stained nitrocellulose strip of the BMZ extract which was electrophoresed in the presence of 0.1 M dithiothreitol and electrophoretically transferred to nitrocellulose. Multiple protein bands are revealed in the extract. *Lane 3* is a nitrocellulose strip of the BMZ extract identical to lane 2 except that it is reacted with EBA antibodies which label only 2 of the protein bands: an intensely stained 290 kD band and a less intensely stained 145 kD band.

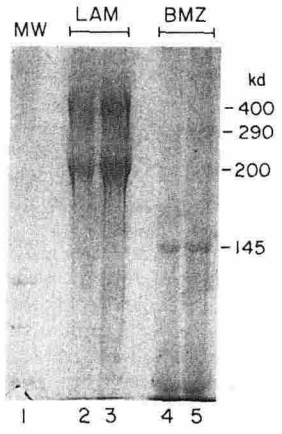

Figure 2. Slab gels of laminin and the BMZ extract stained for carbohydrate. A high-molecular-weight standard (*lane 1*), laminin (*lanes 2 and 3*) and the BMZ extract (*lanes 4 and 5*) electrophoresed into a gel and stained for carbohydrate with metabisulfite-fuchsin. The 400 kD and 200 kD chains of laminin are known to contain carbohydrate and stain positively. Of the extract, the 145 kD EBA band stains the most prominently. The 290 kD EBA chains also react with the stain but much less than the 145 kD chain. Most of the other bands seen on Coomassie blue-stained gels do not react with the carbohydrate stain.

Figure 3. Isolated EBA bands electrophoresed in a second gel and stained for carbohydrate. A high-molecular-weight standard (*lane 1*), 2 bands of the 290 kD EBA chain (*lane 2*), and 1 band of the 145 kD EBA chain (*lane 3*) were cut out of a gel and reelectrophoresed into this gel and stained for carbohydrate with metabisulfite-fuchsin. Using 2 bands of the 290 kD chain to 1 band of the 145 kD chain provides equal staining intensity of the 2 bands.

kD and 145 kD and identified by Coomassie blue staining of polyacrylamide gels, are the proteins that react with monoclonal and polyclonal EBA antibodies in the Western blots. This information allows us to identify the EBA antigen chains in Coomassie blue-stained gels.

When the BMZ extract is incubated with collagenase and the products electrophoresed on a 7.5% polyacrylamide gel, transferred to nitrocellulose paper in a Western blot and reacted with EBA antibodies, it appears that the 290 kD chain but not the 145 kD chain is collagenase sensitive in a concentration-dependent manner (Fig 4). The collagenase fragments generated from the 290,000 chain avidly bind EBA antibodies and have apparent molecular weights of 235,000, 208,000, and 155,000. Parallel experiments using bovine serum albumin as substrate produced no proteolytic fragments either by Western blot analysis with anti-bovine albumin antibody or by silver staining the gels for protein.

Protein eluted from gel slices of the 290 kD and 145 kD bands by the technique of Hunkapiller et al [16] were subjected to labeling with [^{125}I]sodium iodide by the chloramine-T method [18]. However, it was found that only the 145 kD chain could be labeled by this method which is known to rely upon the tyrosine content of the substrate for labeling.

The BMZ extract is prepared in the presence of a reducing agent. However, disulfide bonds apparently re-form during the subsequent preparative steps, since many more protein bands enter the gels when the protein solution is made 0.1 M in dithiothreitol immediately before electrophoresis and boiled for 3 min. Moreover, reduction of the samples prior to electrophoresis is required for the 2 EBA chains to enter the gel (Fig 5). This implies that the native EBA antigen molecule is too large to enter the gel, has a molecular weight above 800,000, and that the EBA antigen chains are most likely linked by disulfide bonds.

Cleveland maps of the 290 kD and 145 kD EBA antigen chains were resistant to cleavage with chymotrypsin in amounts from 0–5.0 μg/lane. In contrast, the 145 kD band incubated with staphylococcal V-8 protease produced multiple proteolytic fragments with 0.0025–2.5 μg of the enzyme (Fig 6). Although only the 145 kD band was placed in lanes on the second gel and reelectrophoresed in the presence of low amounts of proteolytic enzyme (0–0.0025 μg), bands of 290 kD and higher appear in the second gel. This suggests that the 145 kD chains reaggregate to form oligomers of 200 kD and higher, including the 290 kD band, within the lanes of the stacking gel and are subsequently resolved in the separating gel (Fig 6). The appearance of bands of molecular weights higher than 145,000 and 290,000 as well as other intermediate molecular weight bands (i.e., 239,000, etc.) is not unexpected for mixtures of glycoproteins which are known to reaggregate on reconcentration to produce oligomers under transport conditions in denaturing solvents [19–21].

V-8 protease treatment of the 290 kD chain generated 2 cleavage products: a 145 kD fragment and a 79 kD fragment (Fig 7) at relatively high protease concentrations. Both the 290 kD and 145 kD EBA chains had 79 kD proteolytic fragments. Attempts were made to identify antibody binding sites on the EBA antigen chains

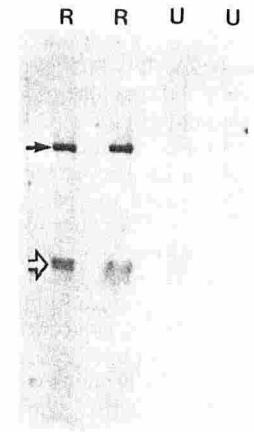

Figure 5. A Western blot of the BMZ extract electrophoresed with (*lanes R*) and without (*lanes U*) the presence of dithiothreitol and reacted with 2 different EBA patients' sera. The EBA 290 kD and 145 kD chains are only revealed when the extract is electrophoresed under reducing conditions. (*lanes R*).

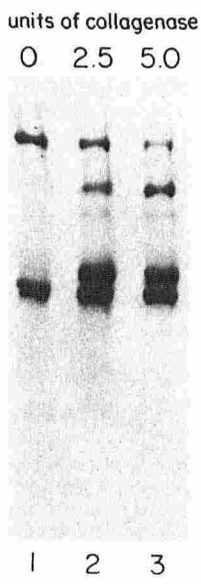

Figure 4. Degradation of the 290 kD EBA band with collagenase. The BMZ extract treated with boiled bacterial collagenase (*lane 1*), 2.5 units of collagenase (*lane 2*), or 5.0 units of collagenase (*lane 3*), electrophoresed on a 7.5% polyacrylamide gel and reacted in the Western blot with EBA antibodies. There is progressive digestion of the 290 kD chain and the generation of proteolytic fragments while the 145 kD chain is resistant to collagenase.

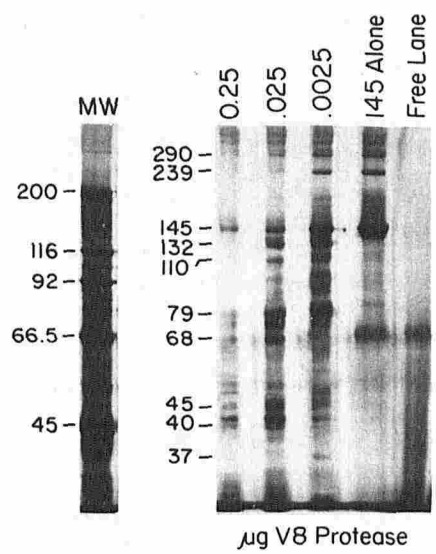

Figure 6. A silver-stained 7.5% polyacrylamide gel of a limited proteolytic digest of the 145 kD chain with staphylococcal V-8 protease. There are multiple fragments generated below 145 kD, but there are also protein bands seen above 145 kD that are not seen in the *Free Lane*. The *Free Lane* and lane without protease (*145 Alone*) also show a 68 kD artifact of unknown origin.

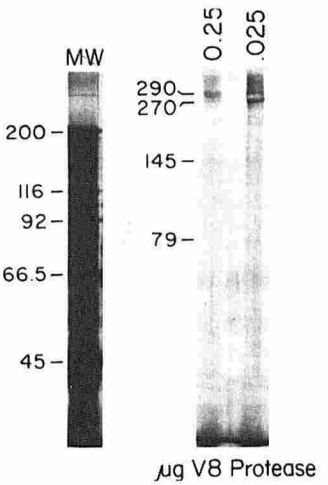

Figure 7. A silver-stained 7.5% polyacrylamide gel of a limited proteolytic digest of the 290 kD chain with staphylococcal V-8 protease. At equal protease concentrations, relatively few breakdown products are generated from the 290 kD chain when compared with the 145 kD chain (Fig 6). However, 145 kD and 79 kD fragments are generated.

by performing Western immunoblots on V-8 protease Cleveland maps and reacting the blots with polyclonal and monoclonal anti-EBA antigen antibodies. However, these attempts were unsuccessful since none of the proteolytic fragments (as revealed by silver stains of the maps) was capable of reacting with antibody in the Western blots (data not shown).

DISCUSSION

The EBA antigen is a normal constituent of the BMZ at the dermal–epidermal junction of human skin. Although the function of this BMZ component is not known, the fact that a subepidermal blistering disease is associated with autoantibodies directed against the EBA antigen and that the blister separation occurs at the site of antigen–antibody complexes suggests that it may play a role in epidermal-dermal adherence in human skin. The solubility properties of the EBA antigen have inhibited its purification to date. However, in these studies we have partially characterized the EBA antigen and provided evidence that it is probably a glycoprotein containing collagen-like domains. The 290 kD chain may have a low tyrosine content which would account for its inability to be iodinated in the presence of chloramine-T. Collagens often have low tyrosine contents and are difficult to label with sodium iodide by the chloramine-T method. Another possibility is that the tyrosine could be hindered by the folding of polypeptide chains and stabilization of disulfide bonds such as occurs with type VI collagen. The 290 kD EBA chain is also collagenase sensitive, but the 145 kD chain is not. The 145 kD chain is easily labeled with [^{125}I]sodium iodide by the chloramine-T reaction. Taken together, these results would suggest that the 290 kD chain is collagenous in nature while the 145 kD chain is noncollagenous.

Multiple bands were generated when the 145 kD protein was subjected to staphylococcal V-8 protease (Fig 6). One of the bands migrating at 79 kD was also seen in the proteolytic digest of the 290 kD chain (Fig 7). Many of the other 145 kD chain breakdown products (M_r = 132,000, 110,000, 68,000, 45,000, and 37,000) may not be EBA antigen-related molecules. This is suggested by the fact that the 145 kD band is much more prominent than the 290 kD band on Coomassie blue-stained gels while in the Western blots the 290 kD band is more heavily labeled. Thus, there are apparently many more anti-EBA antigen binding sites in the 290 kD band than in the 145 kD band.

When the 145 kD chain is placed alone into a lane of the second gel, bands that are larger than 145 kD appear on the gel even in the presence of protease (Fig 6). From previous experiments, we know that we cannot extract the EBA antigen from skin without reducing agents. However, when these agents are removed by dialysis there is re-formation of disulfide bonds and the addition of a reducing agent is again needed before the extract is electrophoresed in order for the EBA chains to enter the gel and be visualized in the Western blots (Fig 5). Therefore, it is probable that the EBA chains are highly cross-linked by disulfide bonding and tend to re-form these bonds when they are broken even in the presence of protease and 2% SDS.

This study shows that the EBA antigen has carbohydrate and collagen-like domains. However, the relationship between the major 290 kD and minor 145 kD chain is still not clear. It is unlikely that the 290 kD band is a dimer composed of two 145 kD chains, since the 145 kD band appears to contain more carbohydrate and is less sensitive to collagenase than the 290 kD subunit.

Some of the non-EBA antigen proteins in the 145 kD band could re-form bonds in the second gel and account for the observed high-molecular-weight bands. However, it is interesting that one of the most prominent re-formed bands migrates at 290 kD. Further, limited proteolysis of both the 290 kD and 145 kD chains produces a common 79 kD breakdown product. Both the 290 kD and 145 kD bands are recognized by antibodies in the sera of EBA patients and by a monoclonal antibody. All of these antibodies, whether polyclonal or monoclonal, label the 290 kD chain more heavily than the 145 kD chain in Western blots. These observations provide evidence that the 290 kD and 145 kD chains do have domains in common, but that the 290 kD chain is not a dimer of 2 identical 145 kD chains. It is more likely that the 290 kD chain is a polymeric protein containing both collagenase-sensitive and collagenase-insensitive domains.

The structure of the native EBA antigen is not known and the solubility properities of the BMZ extracts developed to date have prohibited purification of the EBA antigen. Our studies indicate that the EBA antigen is a large matrix molecule with a complex structure, contains carbohydrate, and has both collagenous and noncollagenous domains. This type of structure would be consistent with the structures of other matrix molecules at the BMZ such as type IV collagen, which has carbohydrate domains, helical collagenous segments, nonhelical flexible regions, and a 7-S collagenase-resistant domain [22–24].

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